84. **Kiyoshi Yoshimura and Minoru Morita** : Studies on Invert Soaps. II.[1] Colorimetric Determination of Benzalkonium Chloride using Phosphomolybdic Acid.

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Of late, the very dilute solution of invert soaps are being used more and more. Up to now, there have been reported various colorimetric methods for its determination. In 1938, Hartley and Runnicles[2] developed a colorimetric method for determining quaternary ammonium cations by using the fact that the color of sulfophthalein indicators was displaced to the alkaline side by paraffinic chain (quaternaries). A few years later, Auerbach reported a method in which quaternary dye salt was extracted with ethylene dichloride[3] or with benzene,[4] and the color intensity of the extracted solution measured colorimetrically. In 1947, Colichiman[5] described the modification of Auerbach's method and Flotow[6] proposed a colorimetric method involving the precipitation of quaternary ammonium salt with potassium dichromate. Recently, Aoki and Iwayama[7] reported on a colorimetry for the estimation of benzalkonium chloride in solution which made use of the principle that blue precipitate is obtained from benzalkonium chloride with ammonium thiocyanate and cobalt chloride and that it can be extracted with chloroform. However, there still is needed a method which is capable of estimating a small amount of it, rapidly yet accurately. For this purpose, experiments were carried out to establish a simple method of assay and it was found that benzalkonium chloride gives precipitate on additoin of phosphomolybdic acid. In this method, precipitate thus formed is dissolved in warm acetone and the blue color produced after addition of stannous chloride is measured colorimetrically. The precipitation was complete in strong acid medium, though not in a weak acidity, and it did not make significant difference in its precipitation whether a little excess or too much of a reagent is used. In addition, the solubility of the precipitate in water increased as temperature became higher. Therefore, it was reacted under cooling with a mixture of ice and water and also water cooled in the same way was used for washing the preipitate. On the other hand, the time necessary for reaching the maximum intensity of color varied with the amount of stannous chloride and with temperature. As a result, 5 cc. of 1% stannous chloride in 3*N* HCl and 10 cc. of 3 *N* HCl were used, the colored solution was maintained at 25°. By this means, the color was of maximum intensity 80 minutes after shaking and then the maximum intensity of color was stable for at least one hour if kept at 25°.

Experimental

Reagent—(1) 4 *N* H_2SO_4. (2) 3 *N* HCl. (3) 2% Phosphomolybdic acid solution : A mixture of phosphomolybdic acid and water (2 : 100) was shaken vigorously, filtered, and the filtrate thus

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[1] Part I : K. Yoshimura, M. Morita, "Studies on Invert Soaps. I. Gravimetric Determination of Benzalkonium Chloride with Phosphotungstic Acid," Bull. Hyg. Lab. (Tokyo), **73** (in press).

[2] G. S. Hartley, G. E. Runnicles : Proc. Roy. Soc. (London), **168**, 420 (1938).

[3] M. E. Auerbach : Ind. Eng. Chem., Anal. Ed., **15** 492 (1943).

[4] M. E. Auerbach : *Ibid.*, **16**, 739 (1944).

[5] E. L. Colichiman : Anal. Chem., **19**, 430 (1947).

[6] E. Flotow : Pharm. Zentralhalle, **83**, 181 (1942).

[7] M. Aoki, Y. Iwayama : "Test Methods for Drugs," Ministry of Health and Welfare, Japan, **4**, 51 (1954).

obtained used as the reagent. (4) 1% $SnCl_2$ solution: 1% $SnCl_2 \cdot 2H_2O$ solution in $3N$ HCl. (5) Acetone. (6) Standard solution: 10% solution of benzalkonium chloride was diluted with water to make about 1% solution, the amount of nitrogen in 5 cc. of that solution was determined 3 times by the Kjeldahl method, and the quantity of benzalkonium chloride was calculated by multiplying the amount of nitrogen by 25.524 (average molecular weight of benzalkonium chloride was taken as 357.33).

Preparation of Calibration Curve Solutions containing 100～300 γ/cc. were prepared from 1% solution, 5 cc. portion of the solution was each pipetted into a test tube, and the color was developed as described in the procedure beginning "5 cc. of $4N$ H_2SO_4 is added and cooled with a mixture……". Calibration curve was made by plotting absorbancy against concentration of benzalkonium chloride on a section paper. All measurements were made at 730 mμ using a Beckman model B spectrophotometer with 1-cm. cuvettes (See Figs. 1. and 2).

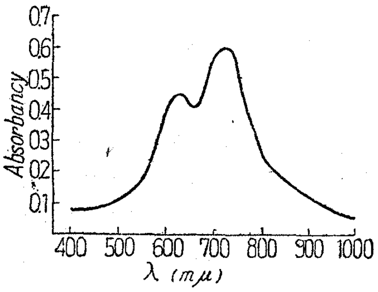

Fig. 1. Absorption Curve

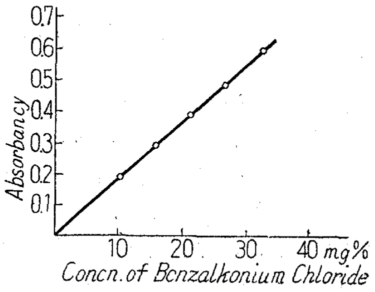

Fig. 2. Calibration Curve

Procedure: Five cc. of the solution containing about 200 γ/cc. is pipetted into a test tube, 5 cc. of $4N$ H_2SO_4 is added, and cooled with a mixture of ice and water. To this mixture is added 5 cc. of 2% phosphomolybdic acid solution, swirled to facilitate precipitation, and the mixture is allowed to stand for a while. Then the precipitate is filtered through a crucible-shaped sintered-glass filter under suction and precipitate is washed with cold water, discarding the filtrate. The precipitate on the filter is dissolved by adding about 10 cc. of warm acetone into a 50-cc. volumetric flask, while applying gentle suction and the filter is washed with about 5 cc. of acetone. To the volumetric flask are added successively 5 cc. of 1% $SnCl_2$ solution in $3N$ HCl and 10 cc. of $3N$ HCl, the whole volume is brought to 50 cc. with acetone, shaken well, and placed at once in a thermostat kept at 25°. After 80 mins. in the thermostat, the absorbancy at 730 mμ is measured, using a blank solution of the reagents which has been treated in the same way as the sample and the quantity of benzalkonium chloride is determined by referring to the calibration curve.

Discussion The precipitation reaction with phosphomolybdic acid, used in this method, is not specific for benzalkonium chloride. By this method, however, a small amount of it can be determined without using any special reagents. As stated before, temperature regulation after adding all reagents in the case of coloration should be strictly observed. Otherwise, it is considered necessary to determine the time sufficient for reaching the maximum intensity of color every time when experiments are made. In the present work, experiments were carried out 18 times using the solution containing 270.3 γ/cc. for the purpose of finding the error due to procedure, the result of which are shown in Table I. From these data, standard deviation was 0.00758 and the error was found to be less than 2%.

TABLE I.

0.399	0.408	0.399	0.405	
0.400	0.406	0.401	0.395	
0.406	0.398	0.416	0.404	x=0.403 df 17
0.397	0.401	0.411		S. D. 0.00758
0.382	0.411	0.407		Error 1.81%

Summary

A new assay method of benzalkonium chloride was established, and it can be

carried out rapidly and accurately. Its principle is to measure the intensity of a blue color that is developed by adding a stannous chloride solution to a solution of the combined substance made from benzalkonium chloride and phosphomolybdic acid.

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85. **Toru Masuda** : Application of Chromatography. XXVIII. On the Formation of FAD in the Culture of *Eremothecium ashbyii**

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In the previous paper[1] the author and co-workers reported on the formation of riboflavin and flavin-adenine dinucleotide (FAD) in the culture of *Eremothecium ashbyii*, and added that studies on the flavin compounds in the mycelium and the culture broth by paper chromatography were under way. In the present paper are announced detailed explanation of such studies and a consideration about the formation of FAD.

E. ashbyii was cultivated under the same conditions as in the previous work and the result was about the same. Samples were collected at 20, 30, 40, 51, 66, 72, and 90 hours after the start of the culture and numbered from one to seven, and various measurements were conducted on them (Table I).

The culture broth separated from the mycelium was applied, as such or after concentrating *in vacuo* to one-tenth its volume, on the starting line of a paper strip and developed with butanol-ethanol-water or with 5% Na_2HPO_4 solution, and also subjected to paper ionophoresis to give the chromatogram and the pherogram shown in Table II. In this test, spots other than that of riboflavin were also detected somewhat clearly in the sample collected after some progress of the culture, but in general, none were so distinct as the spot of riboflavin. It is noteworthy, however, that adenosine was detected in the broth collected after 20 hours but was already absent in the broth collected after 30 hours.

The wet mycelium was extracted with pyridine-methanol (1 : 1) with warming and the extract was developed with butanol-acetic acid-water (4 : 1 : 5) or with 5% Na_2HPO_4 solution, or examined by paper ionophoresis. The resulting spots of flavin compounds were observed by naked eye or under ultraviolet rays and phosphorus was detected by molybdic acid reagent.

On the other hand, an aqueous extract of the mycelium, after addition of ammonium sulfate, was extracted with phenol, the phenol layer was shaken with ether and water, and the aqueous layer was tested as above. However, since in these cases flavin compounds seemed to have been lost considerably during the procedures, the mycelium was extracted with hot water (80°), and after filtering off the separated riboflavin, the filtrate was developed with butanol-ethanol-water (50: 15 : 35) or with 5% Na_2HPO_4 solution, or subjected to paper ionophoresis (Table III). As a result, flavin compounds were hardly lost and all of them could be detected on the paper, and butanol-ethanol-water was found to be the most suitable solvent.

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* This constitutes a part of a series entitled "Application of Chromatography" by Satoru Kuwada.

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[1] This Bulletin, **3**, 375 (1955).